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Responses of Photosynthetic Efficiency and Ascorbate Peroxidase Induced by Salt Stress in Rice (*Oryza sativa* L.)

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We investigated changes in photosynthesis and activity of ascorbate peroxidase (APX) that scavenges ROS as responses to oxidative stress induced by salinity in rice (*Oryza sativa* L.). Photosynthetic efficiency of rice leaves, monitored in terms of Fv/Fm, declined with the increase of salt concentration (100-300 mM NaCl). Salinity caused an increase of H_2O_2 in leaves of rice, with an increase of APX activity. Among total APX isoforms, an isoform of stromal-APX 1 in leaves of rice was completely inactivated by 300 mM NaCl, but was not affected by chilling or drought. The results suggest that salt stress acts in quite a different mechanism in relation to the activity of stromal-APX from that of other stresses such chilling and drought. We carried out RT-PCR for analysis of genes expression of APX isoforms as affected by salt stress. The expression of cytosolic *APX* / thylakoid-bound *APX* genes in leaves of rice exposed to salt stress was increased, while stromal *APX* gene expression rapidly declined.

Key words : Rice, salt stress, ascorbate peroxidase (APX), stromal *APX* gene, Fv/Fm

Introduction

Abiotic stress such as drought and salinity causes considerable damages to crop worldwide [4,8,31]. When plants are exposed to abiotic stresses, they experience functional disorder of metabolism including oxidative stress, osmotic stress and ionic imbalance [13,20,32]. Salinity induces oxidative stresses in plants by increasing reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical [11,26]. The excessive generation of ROS damages to photorespiratory pathway and electron transport within chloroplast and mitochondria. Generally, when plants are in the growing state ROS generation within cell is less than 240 uM $O_2^{\cdot-}$, and in the stable state H_2O_2 in chloroplast is about 0.5 uM [3]. Under abiotic stress such as salinity, however, ROS generation is higher than 720 uM and 15 uM for $O_2^{\cdot-}$ and H_2O_2, respectively, which disturb homeostasis of cells. Hydrogen peroxide, one of ROS, generates more active hydroxyl radicals by Haber-Weiss reaction [21]. These free radicals are so active that they may damage macromolecules such as DNA, proteins and lipids in the cell. Therefore, scavenging of hydrogen peroxide is very important in maintaining photosynthesis in chloroplasts and in keeping metabolism balance within cytoplasm.

Ascorbate peroxidase (APX) is one of enzymes that play an important role in eliminating H_2O_2 not only in chloroplast but also in cytoplasm. APX is a primary H_2O_2 scavenging enzyme that has high substrate specificity for ascorbate [3]. APX can easily eliminate H_2O_2 with high affinity in the cytosol, chloroplasts (stroma and thylakoid membrane) and other subcellular locations (microbody) [9,16,37]. These APX isoforms have been separated from spinach chloroplasts, tea leaves and plastids of tobacco, followed by many studies on their enzymatic/molecular properties [6,29,35]. The significant differences of those isoforms are apparent in their physico-chemical and kinetic properties. Compared to cytosolic form, chloroplastic isoforms (tAPX and sAPX) have very short life time in the culture medium without ascorbic acid (AsA) and their sensitivity to thiol reagents as well as to inhibitors (such as hydroxylamine) is much higher than their cytosolic counterparts [6,7]. ROS metabolism in a particular compartment can effect the different cellular compartments. The application of light stress to Arabidopsis has been reported in the induction of cytosolic. But not induce chloroplastic ROS removal enzymes [10,18,19,33]. Several studies showed that activity of cytosolic/chloroplastic APX is important in protecting plant cells from oxida-

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tive stress, which is one of many dysfunctions caused by salt stress [3,40]. We investigated changes in photochemical efficiency, activity of ascorbate peroxidase (APX) as well as its mRNA expression induced by oxidative stress in rice leaves that had been exposed to salinity. The observations suggested that a salinity-induced inactivation of an APX isoform might play a critical role in the tolerance mechanism of rice plants to salt stress.

Materials and Methods

Plant material and induction of salt stress

Surface-sterilized seeds of rice (*Oryza sativa* L. cv. Dongjin) were germinated in water for 5 days at 25°C under dark conditions. After germination, seedlings were grown in a vermiculite medium with Hyponex fertilizer for 15 days in a growth chamber maintained at 25°C and 70% RH with a photoperiod of 14 hr (200 μmol quanta m^{-2} s^{-1}). Then, the seedlings were exposed to various concentrations of NaCl (100, 200, and 300 mM) for 24 hr.

Chlorophyll fluorescence analysis and estimation of H_2O_2 level

Chlorophyll fluorescence emitted from the upper surfaces of leaves was measured using chlorophyll fluorescence measuring system (PAM-2000, Walz, Germany). F_o and F_m was measured from the dark-adapted leaves with an excitation light intensity of 200 μmol quanta $m^{-2}s^{-1}$ and photosynthetic efficiency of PSII (F_v/F_m) was calculated as described by Hwang et al. [15]. H_2O_2 levels in leaves and roots of rice were measured according to the modified method of Lee et al. [21]. Leaves and roots of rice (1 g) were homogenized in 100 mM sodium phosphate buffer (pH 7.0). The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000× *g* at 4 °C for 20 min. An aliquot (0.5 ml) of supernatant was mixed with 2.5 ml of peroxide reagent consisting of 83 mM sodium phosphate (pH 7.0), 0.005% (w/v) o-dianisidine, 5 unit peroxidase (Sigma, USA) followed by incubation at 30 °C for 10 min. The reaction was stopped by adding 0.5 ml of 1 N perchloric acid and centrifuged at 3,000× *g* for 5 min. The absorbance of the supernatant was read at 436 nm, which was compared to the extinction of a H_2O_2 standard.

Ascorbate peroxidases assay

Leaves and roots of rice (1 g) were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000× *g* at 4°C for 20 min. APX activity was determined following the method of Lee et al. [21]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM hydrogen peroxide and the suitable volume of enzyme extract. The H_2O_2 dependent oxidation of ascorbic acid was followed by monitoring the decrease of absorbance at 290 nm assuming an absorption coefficient of 2.8 mM cm^{-1}. Protein content was measured according to the method of Lowry et al. [24] with bovine serum albumin (BSA) as a standard.

Isolation of chloroplast

Chloroplast was isolated from rice leaves as modified by Bruce et al. [5]. Fresh leaves (10g) were homogenized in a Waring blender with 15 ml of homogenizing buffer containing 0.35 M sorbitol, 25 mM HEPES-KOH buffer (pH 7.8), 2 mM EDTA, 5% soluble PVP (w/v) and 0.15% BSA with addition of 20 mM ascorbate. The homogenate was filtered through four layers of cheesecloth and centrifuged at 2,000× *g* for 1 min. The pellet was suspended in 25 mM HEPES-KOH buffer (pH 8.0) containing 2 mM ascorbate. Five milliliter of suspension medium containing 32% (v/v) Percoll (Amershaam Pharmacia Biotech) was layered under the chloroplast suspension at the bottom of the tube. Tubes were centrifuged at 750× *g* for 3.5 min and the pellet of intact chloroplasts was resuspended in 1 ml of suspension medium. Then, after the resuspension mixture was centrifuged at 18,000× *g* for 20 min, the supernatant obtained was used as the soluble fraction and the pellet was used as the membrane fraction.

Electrophoresis and activity gel analysis

Plant extracts containing equal amounts of protein, with the addition of bromophenol blue and glycerol to a final concentration of 10%, were subjected to discontinuous PAGE under non-denaturing, non-reducing conditions essentially as described by Lee et al. [21], except that SDS was omitted and the gels were supported by 10% glycerol. Electrophoretic separation was performed at 4°C for 4 hr with a constant current of 30 mA per gel. Then, the gels were incubated in a solution composed of 50 mM sodium phosphate (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. They were washed in the buffer with gentle agitation for 1 min and submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM Nitro Blue

Tetrazolium (NBT).

Immunodetection of APX-1 by native-western blot

The extracts from leaves of rice were separated in a preparative 10% native-PAGE, and electrotransfered to nitrocellulose membrane filters. Filters were blocked with TBST buffer containing 0.5% Goat IgG, incubated for 2 hr with 1:1,000 dilution of the polyclonal antibody raised to APX-1 isoform, and then washed with the TBST buffer. TBST buffer contained 10 mM Tris-HCl (pH 8.0), 100mM NaCl and 0.05% Tween 20. Antibody reacting protein bands were revealed staining at 0.02% 3, 3′-Diaminobenzidine tetrahydrochloride (DAB).

RNA extraction and RT-PCR

Total RNA was isolated from leaves using TRI-reagent (Sigma) according to the manufacturer's instruction. The first strand cDNA was synthesized from 5 μg of the total RNA with oligo - (dT) primer according to the instruction of MMLV First Strand Kit (Promega). RT-PCR was performed in a total volume of 25 μl containing 5 μl of the first strand cDNA reaction products, 2.5 μl of 10×PCR buffer, 2 μl of dNTP, 10 pmol of primers and 2.5 units of rTaq DNA polymerase (Takara). The primers for cAPX were 5′-CCA AGGGTTCTGACCACCTA-3′ and 5′-CAGTTGAGCATAAC AGCCCA-3′; sAPX primers were 5′-ATCCTGGTTCGTCTT GGATG-3′ and 5′-ACTTCAGCGATCTGGCTCAT-3′; tAPX primers were 5′- AGGGAGGTATTCTACAG GATG-3′ and 5′-CTTTCCGTAGGAGTATTTCG-3′. They were designed on the basis of the published sequences of cytosolic APX (cAPX, NCBI D40423), stromal APX (sAPX, NCBI AB114855) and thylakoid-bound APX (tAPX, www.Tigr.org OsO2g34810) of *Oryza sativa* L. PCR was started with 5 min at 95°C, carried out 30 cycles consisting of 45 s at 95°C, 1 min at 55°C and 72°C, and ended up with 7 min at 72°C (Eppendorf AG 22331 PCR system)

Results

To examine salinity effects on photosynthesis of rice leaves, chlorophyll a fluorescence was measured from leaves that had been treated with NaCl at the concentration from 100 to 300mM for 24 hr. F_v/F_m decreased by 19% with 300 mM NaCl treatment (Fig. 1). Decrease in F_v/F_m ratio was due to the salinity-induced depression of F_v (data not shown), which known is related to the reduction of primary electron acceptor pool of PSⅡ.

During exposure to salt stress, leaves showed elevation of H_2O_2 level, which is the measure of plant capacity to produce ROS in the cytoplasm, in contrast to roots which showed no such response (Fig. 2). The amount of H_2O_2 in leaves increased in a concentration-dependent manner with 200 mM NaCl showing 1.57 fold-increases. The results indicated that degree of oxidative stress induced by salt stress was more apparent in leaves than in roots.

Oxidative stresses caused by the high accumulation H_2O_2 might damage cell components and therefore it can be said that welfare of the cell could depend on the rapid elimination of such a ROS. When the activity of APX in leaves was measured during salt stress, it increased 1.2 fold during treatment of 100 mM NaCl, while no corresponding changes were observed in roots (Fig. 3).

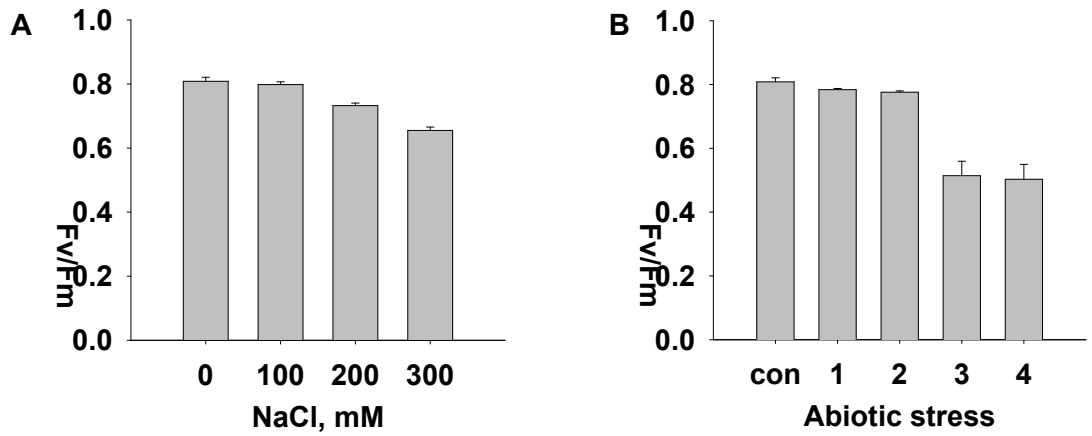

Fig. 1. Photosynthetic efficiency (F_v/F_m) in terms of chlorophyll fluorescence in leaves of rice plants that had been exposed to salt stress (A) or abiotic stresses for 24 hr (B). con, control; 1, 4°C chilling; 2, drought; 3, 10 mM 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU); 4, 0.1mM Methyl viologen (MV). The values represent mean±SD (n=6).

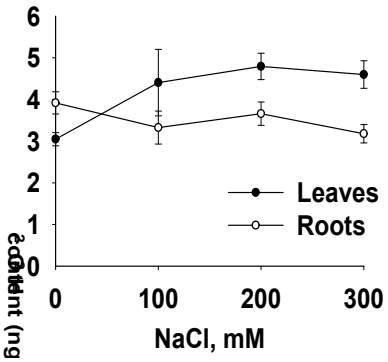

Fig. 2. Changes in H_2O_2 contents in leaves and roots of rice plants that had been exposed to various concentrations of NaCl for 24 hr. The values represent mean±SD (n=6).

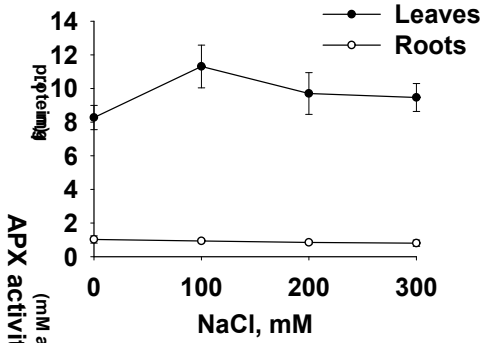

Fig. 3. Activities of ascorbate peroxidase (APX) in leaves and roots of rice plants that had been exposed to various concentrations of NaCl for 24 hr. The values represent mean±SD (n=6).

To understand such tissue-specific activity of APX isoforms during salt stress, we separated them from each other by carrying out electrophoresis. Seven isoforms of APX were apparent on the gel. When leaves were treated with increasing concentration of NaCl, APX-6 showed increasing activity, but contrastingly, activities of APX-1 and APX-5 decreased gradually (Fig. 4). When leaves were treated with 300 mM NaCl, most noticeably, the activity of APX-1 was completely inactivated, indicating an extreme sensitivity of leaf APX-1 to salinity stress. In roots, activities of 4 isoforms (APX-3, APX-4, APX-5 and APX-6) were confirmed to be expressed, but they showed little alterations by salt treatments.

Sensitivity of APX-1 to other abiotic stresses such as drought or chilling was also examined (Fig. 5), but it revealed that APX-1 was not much affected by such treatments, indicating that inactivation of APX-1 was a salinity-specific response of rice leaves. To investigate whether such a salinity-induced inactivation of APX-1 is resulted

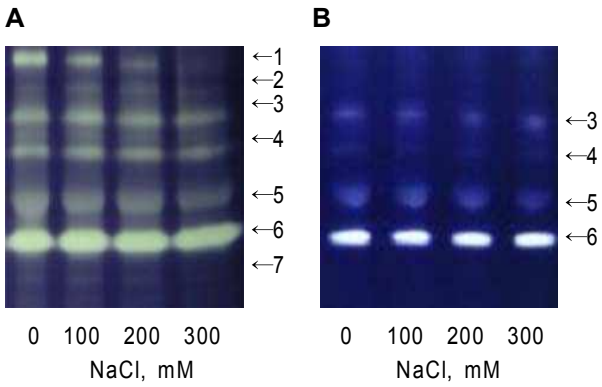

Fig. 4. Native gel stained for the activities of ascorbate peroxidase (APX) in leaves (A) and roots (B) of rice plants that had been exposed to various concentrations of NaCl for 24 hr. The numbers on the right side of photographs represent the respective isoforms of APX.

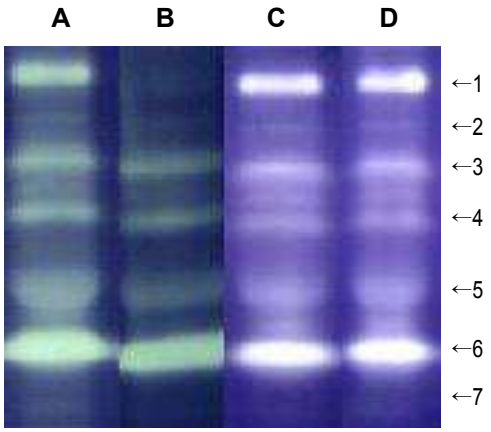

Fig. 5. Native gel stained for the activities of ascorbate peroxidase (APX) in leaves of rice plants that had been exposed to abiotic stresses for 24 hr (lane A, control; lane B, 300 mM NaCl; lane C, chilling; lane D, drought).

from altered expression of APX-1 protein, we carried out Western blot analysis (Fig. 6). When leaves were exposed to 300 mM NaCl, presence of APX-1 protein was not detected.

When APX isoforms of rice chloroplasts were separated into each fraction of membranes and stroma, the stromal fraction revealed activities of 7 isoforms, while the membrane fraction only contained activities of APX-5 and APX-6 (Fig. 7). APX-1 and APX-2 were present only in the soluble fraction, but not in the membrane fraction or in roots, indicating APX-1 belongs to stromal APXs.

To examine any alterations of mRNA expression of APX isoforms as affected by salt stress, we carried out RT-PCR analysis of genes for cAPX, sAPX and tAPX (Fig. 8). With

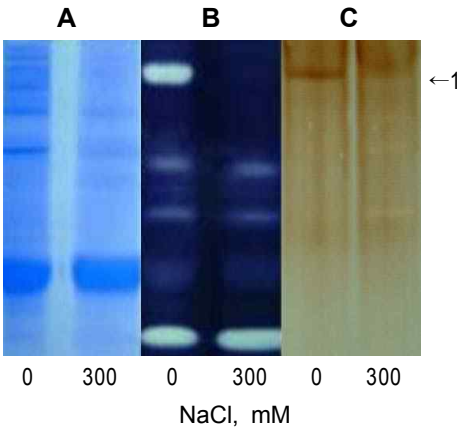

Fig. 6. The loss of the APX-1 isoform in leaves of rice plants. Salt-induced inactivation of APX-1 isoform. After rice plants were exposed to 300mM NaCl for 24 hr, crude extracts were prepared from 50 μg of leaves and analyzed by 10% native-PAGE. A, Coomassie blue staining; B, NBT staining; C, immunodetection with 0.02% DAB.

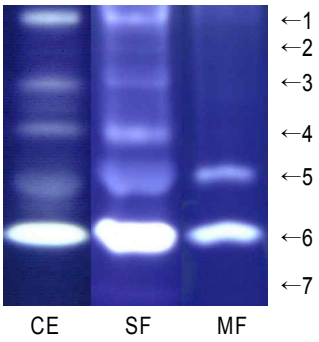

Fig. 7. Compartmentation of ascorbate peroxidase (APX) isoforms in intact chloroplast. 10% native-PAGE gel was stained with NBT (CE, crude extract; SF, soluble fraction; MF, membrane fraction).

increase in the concentration of NaCl, mRNA expression of cAPXs and tAPX gradually increased. Contrastingly, with treatment of 300 mM NaCl, mRNA expression of sAPX showed a marked decline, indicating that those isoforms of leaf APXs act in a different mechanism to salt stress.

Discussion

Salt causes harmful effects on crop plants by generating or accumulating ROS that leads to disturbance of cellular homeostasis, followed by oxidative stress [14,39,43]. There are many reports that ROS generated from salt stress causes problems in the metabolism of chloroplasts [3,22,40]. Photosynthetic apparatus is considered to be a main target

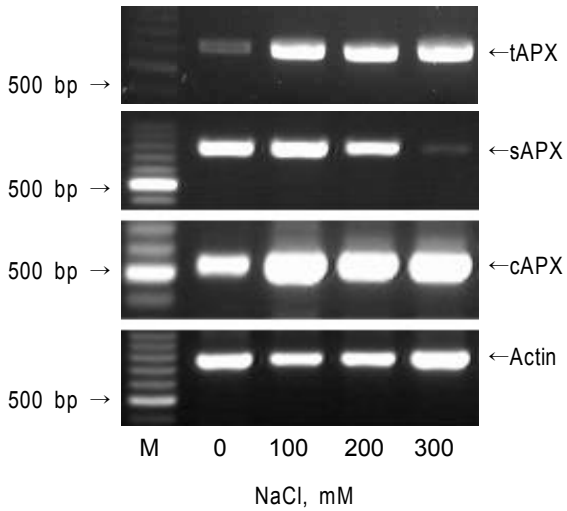

Fig. 8. RT-PCR analysis of thylakoid-bound (tAPX), stromal (sAPX), and cytosolic ascorbate peroxidase (cAPX) in leaves of rice plants that had been exposed to various concentrations of NaCl for 24 hr.

of ROS generated from salt stress.

Salinity-induced F_v/F_m decline in rice leaves that had been observed in the present study shows that salt stress had caused damages to the photosynthetic apparatus. We previously showed that reduction in F_v/F_m ratio (Fig. 1A), was related to oxidative stress [1,32], but which was not related to chilling and drought stresses (Fig. 1B). H_2O_2 is produced under various stress conditions such as water deficit or salinity [12,17,30]. H_2O_2 may be generated in the process of ROS degradation, which is enzymatic or non-enzymatic, and distributed in chloroplasts and cytoplasm. As H_2O_2 is an active oxygen species, without prompt elimination, it generates strong hydroxyl radicals by the transition metal-catalyzed Haber-Weiss reaction. These hydroxyl radicals oxidize most of the cell components according to diffusion-controlled rate and result in cellular damages. Change of H_2O_2 content in plants is considered as a sign to notify ROS generation and its corresponding oxidative stress induction. In rice plants under oxidative stress, amount of H_2O_2 was shown to change, indicating capacity of plants to eliminate ROS in the cytoplasm. While NaCl treatment little influenced the level of H_2O_2 present in roots, that of leaves were markedly increased (Fig. 2).

APX is an enzyme that is primarily responsible for eliminating H_2O_2 and uses AsA as an electron donor when converting toxic H_2O_2 to harmless H_2O and O_2. The increase of APX activity is very important in protecting plants from environmental stresses such as chilling, metal toxicity,

drought and heat because it is involved in eliminating H_2O_2 within cells [6]. The present work showed that leaves of rice plants elevated APX activity in response to salt stress (Fig. 3). APX has been identified as cytosolic (cAPX) and chloroplastic (tAPX, sAPX) forms in plants. Activities of individual APX isoforms are related to increased generation of ROS during salt stress. In addition, their biochemical characterizations show different patterns in terms of properties such as substrate specificity, optimal pH and ascorbate depletion [3,23,38]. When PAGE analysis of leaf APX isoforms was carried out, it revealed seven isoforms and particularly, an increase in APX-6 activity at the NaCl concentration of 300 mM (Fig. 4). Lee et al [21] reported that specific isoforms of rice leaf APX increased their activities during salt stress. In the present study, we demonstrated that the activity of APX-1 decreased with increase in the degree of salt stress, leading to its complete inactivation at the NaCl concentration of 300 mM. It was quite contrasting to other abiotic stresses such as drought or chilling that did not cause corresponding responses (Fig. 5). Those observations suggest that rice leaf isoforms of APXs have specific responses to salinity, different from those to drought or chilling stresses. When proteins were isolated from leaves that had been exposed to salt stress and subsequently subjected to Western blot analysis, APX-1 protein was not immunodetected from leaves that had been treated at the concentration of 300 mM NaCl (Fig. 6). The result suggests a possibility of salinity-induced APX-1 degradation or impaired gene expression. On the other hand, in roots there appeared only four isoforms of APX (APX-3, APX-4, APX-5 and APX-6) and, moreover, salinity-induced alterations were not observed in the activities of APX isoforms (Fig. 4). To find out the location of APX-1, intact chloroplasts were isolated from rice leaves and were separated into membrane fraction and soluble fraction (Fig. 7) using PAGE. APX-1 was only detected in the soluble fraction, not in roots or in membrane fraction. These results indicate that APX-1 is a stromal enzyme. Yamaguchi et al [41] reported that APX isoform with the same molecular mass is located in thylakoid, stroma, mitochondria, and cytosol in leaves of pumpkin.

Cytosolic APX is located in the cytosol of photosynthetic and non-photosynthetic tissue. Although mRNA expression of cAPX is induced by environmental stimulation, its functions are not still clarified. For chloroplastic APX, although partial amino acid sequences were determined, not much informations are available. Recent reports tends to focus on individual APX responses to environmental stresses [27,28,34,42], demonstrating that mRNA expression of cAPX is increased with drought, heat or chemical treatment such as methyl viologen. Asada [3] suggests that cellular location and function of cytosolic APX play an important role in protecting non-photosynthetic cells from oxidative damages. From this study, it was found that mRNA expression of cAPX was increased with salt treatment in leaves of rice plants (Fig. 8). The same trend has been reported earlier in *Nicotiana plumbaginifolia* L. [36]. Plants lacking chloroplastic APX are more sensitive to light and oxidative stress [10,33]. Tanaka et al [40] suggested that increase of chloroplastic APX activity is involved in protecting photosynthesis under high salinity. According to this study, 300 mM NaCl rapidly depressed mRNA expression of sAPX, but expression of tAPX enhanced with the increase of salt concentration. The different levels in the induction of sAPX and tAPX were found in pumpkin under the dark conditions [25]. They suggested that alternative splicing for sAPX/ tAPX mRNAs transcript might be regulated by their developmental state and environmental conditions in the cotyledons of pumpkin. Under photooxidative stress induced by MV, cAPX was more resistant than sAPX [2]. But the enhanced expression of cAPX mRNA is not always translated into the parallel change in the protein level [42]. In the near future, complex mode of integration of ROS signals generated in the cytosol or chloroplast of rice could be revealed through studies on the relationship of mRNA/protein levels of each APX isoform.

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초록 : 벼의 salt stress에 의해 유도된 산화 stress에 대한 ascorbate peroxidase 반응

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Salt stress가 벼 잎 내 광합성 효율과 ascorbate peroxidase (APX) 활성에 미치는 영향을 조사하였다. 염 농도가 증가(NaCl, 100-300 mM) 할수록 Fv/Fm 값이 감소한 반면, H_2O_2 양과 APX 활성은 증가하였다. APX isoforms 중 APX 1 (stromal)은 300 mM NaCl 처리 시 활성이 거의 나타나지 않는 반면, chilling이나 drought 처리 시에는 변화가 없었다. 또한 gel 상에서의 서로 다른 APX isoforms의 활성이 유전자 발현에서도 확인이 되는지를 알아보기 위해 RT-PCR을 수행하였다. 구획별 APX isoforms의 RT-PCR 수행 결과, cytosolic/thylakoid bound *APX* 발현양은 증가한 반면, stromal *APX* 발현은 매우 감소하였다. 이러한 실험 결과는 salt에 의해 구획별로 APX 활성이 영향을 받음을 나타낸다.